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SCHOOL OF AVIATION MEDICINE
RANDOLPH AIR FORCE BASE, TEXAS

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MODIFICATIONS OF THE REDDY METHOD FOR THE ESTIMATION OF 17-HYDROXYCORTICOIDS IN BLOOD

A procedure evolved from modifications of existing technics for the estimation of total 17-hydroxycorticoids in blood has been described. The precision of the method based on the average difference between 95 sets of duplicates indicated a standard deviation of 5.48 and a coefficient of variation of 10.38 percent. Essentially, quantitative recoveries (mean, 98 percent) were obtained by adding hydrocortisone to protein-free plasma filtrates; relatively poor recoveries (mean, 65 percent) resulted from addition of hydrocortisone to untreated plasma samples. A range of 9 to 82 gamma percent was found in a series of 52 subjects, with a mean of 41.2 gamma percent.

In 1950, Porter and Silber (1) described a quantitative color reaction for 17, 21-dihydroxy-20-ketosteroids. From this development, a wide variety of procedures for the determination of plasma and urinary corticosteroids has evolved. Essentially, all such procedures involve three phases: extraction, purification, and colorimetric evaluation. Borth (2) recently presented a critical review of urinary methods in use at the present time. Methods for the determination of blood corticoids have been reviewed by Gold (3).

Alterations in plasma steroid levels appear more meaningful than urinary steroid changes in providing a quantitative interpretation of adrenocortical function in man. Blood studies have considered primarily the free (unconjugated) steroids in peripheral blood.

In the search for a more definitive reflection of the rate of adrenocortical secretion, Reddy et al. (4) developed a butanol extraction procedure for the determination of plasma total 17-hydroxycorticoids (17-OHCS). These workers considered the term "total" justified on the following bases: (a) the earlier use of butanol for the extraction of water-soluble conjugated estrogens and 17-ketosteroids (5); (b) the spectral curve similarities obtained between known and unknown extracts when using the relatively specific Porter-Silber reaction (4); and (c) the correlation of steroid secretion and excretion rates with known states of adrenal cortical function (4, 5). The method devised by Reddy included steroid extraction from plasma with butanol and sodium sulfate; purification with sodium carbonate; evaporation of the solvent; and color development with a sulfuric acid-ethyl alcohol-phenylhydrazine reagent.

The need for 10 ml. of plasma in the Reddy method led other investigators (6) to develop a micromethod for total 17-OHCS. Utilizing 5 ml. of plasma per assay, these workers reported values that were not substantially different from those found by the Reddy technic.

When work was undertaken in this laboratory to incorporate the determination of blood 17-OHCS in a current study on physiologic changes evidenced by aircrew personnel, several technical difficulties were encountered. This paper is concerned with these problems and a determination method based on their solution.

TECHNICAL CONSIDERATIONS

Protein precipitation

Difficulty was encountered in initial attempts to duplicate the micromethod of Brancaccio et al. (6). Values averaging 10 gamma percent were obtained, in contrast to a mean of 34 gamma percent reported by the Italian workers.

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TABLE I

Comparison of 17-hydroxycorticoid extraction and purification procedures

Sample No.	10-min. extraction with butanol 5-min. purification		10-min. extraction with butanol 10-sec. purification		5-min. extraction with butanol 5-min. purification	
	Mean*	S.D.	Mean*	S.D.	Mean*	S.D.
1	55.6	5.2	20.6	4.3	49.4	5.5
2	46.3	7.7	21.2	5.2	45.5	9.0
3	51.6	8.8	28.9	3.8	54.5	7.7
4	54.2	9.8			45.9	6.3
5	57.5	5.3	31.5	18.9		

*Mean of 4 determinations, expressed in gamma percent.

Only after incorporating a stepwise, thorough mixing of plasma with the protein-precipitating reagents were acceptable values obtained. The technic employed is described in detail later in this paper.

Steroid extraction

In an attempt to achieve maximal extraction of blood steroids into butanol, a comparison was made between the conventional 5-minute extraction period and a 10-minute period. The longer period produced slightly higher steroid values (table I) and, for this reason, has been incorporated in the procedure used in this laboratory. Extraction periods longer than 10 minutes produced no increase in steroid values.

Extract purification

To remove nonspecific chromogens, Reddy et al. (4) added anhydrous sodium carbonate to the butanol extract, and the mixture was "thoroughly shaken." Brancaccio et al. (6) incorporated an apparently arbitrary period of 5 minutes for shaking the extract. To clarify requirements for purification, a comparison was made between the use of a 5-minute mechanical shake of the carbonate-treated butanol extract and a vigorous 10-second digital shake. The 5-minute shaking period proved to be the more effective procedure (table I). Shaking in excess of 5 minutes did not provide additional purification.

Drying of extract

Brancaccio et al. (6) stated that the butanol extract was dried in a "tube" at 80° to 85° C., while Reddy et al. (4) recommended drying in an Erlenmeyer flask suspended in a 90° to 95° C. water bath. Efforts with these evaporation technics proved either unsuccessful or excessively time-consuming. Dryness could not be achieved without complicated aspiration apparatus to compensate for reflux tendencies. Use was then made of ground glass – rimmed weighing bottles, 50 mm. high and 40 mm. in diameter. By a technic described later in this report, 21 samples can be dried together in 17 to 22 minutes. Subsequent incubation of these samples was carried out in the same bottles, thus affording greater accuracy as well as simplified methodology.

Additional experiments proved that temperatures as low as 60° C. produced mean values virtually identical to those for 85° to 87° C. and 80° to 82° C. (table II). The 80° to 82° C. range was employed because of the better duplication of values obtained on replicates dried at that temperature.

Sample storage

Since a complete assay run of 16 unknowns, 2 standards, and 2 blanks required approximately 6 hours, it was desirable to determine if the samples could be processed to the protein-free filtrate (PFF) stage, then frozen, and the determination completed later. Two complete assay runs could then be completed from the PFF

TABLE II

Effect of temperature in the evaporation of butanol extracts of 17-hydroxycorticoids

Temperature (° C.)	Time for evaporation (min.)	17-hydroxycorticoids	
		Mean*	S.D.
95°–97°	8–10	33.5	9.5
85°–87°	14–16	47.0	6.9
80°–82°	17–22	46.5	2.9

*Mean of 5 determinations, expressed in gamma percent.

state in a single working day. Although slightly higher levels were obtained from an uninterrupted plasma assay (table III), the greater convenience associated with freezing at the PFF state warranted its incorporation in the routine analysis of large numbers of blood samples.

Plasma versus serum

In associated blood electrolyte studies, the use of serum was advantageous. A comparative study of steroid levels in plasma and serum revealed no systematic differences (table IV).

REAGENTS

It is mandatory that reagents of highest grade be employed in this procedure. Inferior grade or contaminated lots of butyl alcohol, sulfuric acid, ethyl alcohol, or phenylhydrazine produce excessively high blanks and erratic standard curves. The reagents listed in this paper have proved satisfactory when purified and utilized as indicated.

1. Zinc sulfate, 10 percent. Dissolve 100 gm. of $ZnSO_4 \cdot 7H_2O$ (reagent grade) in distilled water and dilute with water to 1,000 ml. in a volumetric flask.

2. Sodium hydroxide, 0.5 normal. Dissolve 20 gm. of NaOH (U.S.P. pellets) in distilled water and dilute with water to 1,000 ml. in a volumetric flask.

3. Sulfuric acid, 50 percent. Add 100 ml. of concentrated H_2SO_4 (Fisher reagent grade) to 100 ml. of distilled water.

4. Sodium sulfate, anhydrous, granular (reagent grade).

TABLE III

Effect of freezing protein-free plasma filtrates

Sample No.	17-hydroxycorticoids (gamma percent)	
	Plasma*	PFF†
1	67.4	75.0
2	63.5	59.0
3	61.2	63.5
4	39.5	27.0
5	37.5	31.2
6	18.8	14.5
7	27.0	31.2
8	86.2	79.5
9	72.8	68.2
Mean	52.7	49.9

*Complete assay was carried out on fresh plasma samples.

†Assay was carried out on fresh plasma to step 3 of extraction procedure (protein-free filtrate), then filtrate was frozen and assay completed 72 hours later.

TABLE IV

Comparison of 17-hydroxycorticoid levels in plasma and serum

Sample No.	17-hydroxycorticoids (gamma percent)	
	Plasma	Serum
1	52.2	50.0
2	34.0	41.0
3	45.5	47.5
4	50.0	50.0
5	31.8	36.2
6	72.8	72.8
7	54.5	52.2
Mean	48.7	49.9

5. n-Butyl alcohol (Mallinckrodt analytic reagent grade). This reagent is purified according to a procedure described by Reddy (7).

6. Sodium carbonate, anhydrous (reagent grade).

7. Ethyl alcohol (absolute pure EtOH, U. S. Industrial Chemicals Co.). Redistill before use.

8. Sulfuric acid, 62 percent. Add 620 ml. of concentrated H_2SO_4 (Fisher reagent grade) to 380 ml. of distilled water.

9. Phenylhydrazine hydrochloride (Baker's purified). This reagent is purified according to the procedure described by Peterson et al. (8).

10. Phenylhydrazine reagent. To 5 ml. of 62 percent sulfuric acid is added 87.5 mg. of phenylhydrazine

hydrochloride. This reagent is prepared fresh before each use.

11. Blank reagent. Four parts of 62 percent sulfuric acid are added to one part of ethyl alcohol.

12. Hydrocortisone standard. Ten mg. of hydrocortisone is dissolved in 100 ml. of n-butyl alcohol. This stock solution remains stable for at least 6 months in the refrigerator. A dilute working standard is prepared by diluting 2 ml. of the stock solution to 100 ml. with n-butyl alcohol (2 gamma per milliliter).

PROCEDURE

Extraction

1. To 5 ml. of plasma or serum in a 15 ml. ground glass–stoppered centrifuge tube, 1.0 ml. of 10 percent $ZnSO_4$ is added. The mixture is shaken vigorously for 30 seconds, and an additional 1.5 ml. of $ZnSO_4$ solution is added. The mixture is again shaken vigorously for 30 seconds. An identical additive procedure is carried out with 2.5 ml. of 0.5 normal NaOH.

2. After centrifugation for 30 minutes at 2,500 r.p.m., the supernate is decanted into another tube and recentrifuged for 10 minutes at 2,500 r.p.m.[1]

3. The supernate is decanted into a 25 ml. ground glass–stoppered tube[2] containing 3 drops of 50 percent H_2SO_4. (If it is necessary to interrupt the procedure, the sample may be frozen at this point.) Two grams of sodium sulfate are added, and the tube is shaken vigorously until the Na_2SO_4 has dissolved.

4. Five ml. of n-butyl alcohol are then added, and the mixture is agitated at maximal speed on a mechanical shaker[3] for 10 minutes.

5. After centrifugation for 10 minutes at 2,500 r.p.m., as much of the butanol supernate as possible is transferred to a 15 ml. ground glass–stoppered centrifuge tube. (Delicate control of aspiration is effected by the use of a fixed 20 ml. syringe attached by rubber tubing to a 5 ml. pipette.)

Purification

1. To the butanol extract is added 0.15 gm. of sodium carbonate, and the tube is agitated at high speed on the mechanical shaker for 5 minutes.

2. The mixture is allowed to stand for 5 minutes and is then centrifuged for 5 minutes at 2,500 r.p.m. A 4 ml. portion of the extract is transferred into a weighing bottle.

3. The contents of 21 of these bottles are then dried simultaneously in the following manner:

By use of an electric hot plate, water in a round, stainless steel pan (12½ inches upper inside diameter, 9 inches lower inside diameter, 5 inches depth) is maintained at 80° to 82° C. A sheet metal cover[4] of two circular layers, held 1⅛ inches apart by metal screws, is placed on the pan. The upper layer, the rim of which fits flush against the flange of the pan, is 14⅝ inches in diameter and contains 22 holes, each 1⅞ inches in diameter. The lower circular layer, 12 inches in diameter, contains 22 holes (1 inch in diameter) situated directly under the larger holes of the upper layer. A seal to the pan is afforded by rubber tubing fitted around the rim of the lower layer. Weighing bottles, placed in each of 21 upper holes, completely cover the lower holes. A thermometer is placed in the other opening. Water level is maintained just below the base of the weighing bottles.

The entire setup is placed in a closed hood, with the hood fan operating throughout the drying period. A small household fan, located in a corner of the hood, is turned on after the bottles have been on the water bath for a 4-minute period. Air from the fan is directed over different groups of bottles by rotating the pan a quarter-turn each 4 minutes. This equipment is illustrated in figure 1.

[1]Second centrifugation is essential to provide adequate supernate.

[2]Prepared by W. O. Beentech, SAM Research Shops, from glassware supplied by Corning Glass Works, Cat. No. 6560.

[3]Model CC, wrist-action shaker, Burrell Corp., Pittsburgh, Pa.

[4]Designed by F. V. Gerlich, SAM Research Shops.

FIGURE 1

Equipment utilized for evaporation of butanol.

Colorimetry

1. When evaporation is complete, 2.5 ml. of the blank reagent are added directly to each weighing bottle, and ground glass–stoppers are inserted. The contents are mixed thoroughly by digital rotation of the bottles.

2. The bottles are placed in a water bath at 60° C. for 20 minutes, then cooled in running tap water for 5 minutes.

3. The content of each bottle is pipetted into a 10 mm. Corex cuvette and read in a Beckman DU spectrophotometer at a wave length of 410 mμ, against a water blank.

4. Then 0.1 ml. of phenylhydrazine reagent is added to each bottle, the cuvette content returned thereto, and the bottles reincubated at 60° C. for 20 minutes. They are again cooled, the contents poured into the same cuvettes as before, and a second reading made at 410 mμ, against a water blank. With meticulous care, the required transfer of samples between bottles and cuvettes can be carried out without loss of fluid.

5. In each routine analysis of 16 plasma samples, two 5 ml. aliquots of distilled water are carried through the entire procedure to serve as reagent blanks, and two 5 ml. samples of butanol containing 2 gamma and 4 gamma of hydrocortisone, respectively, serve as standards.[5]

Calculations

A = reading after phenylhydrazine addition.

B = reading before phenylhydrazine addition.

a = A − B (sample).

b = A − B (standard).

c = A − B (reagent blank).

a − c = corrected optical density (C.O.D.) of the unknown sample.

b − c = C.O.D. of standard (then equated to 1 gamma).

$$\text{Gamma of hormone present in 100 ml. of plasma} = \frac{\text{C.O.D. sample}}{\text{C.O.D. standard}} \times 25.$$

Sample calculation:

Sample		*Standard (1.6 gamma)*		*Reagent blank*	
A	.191	A	.049	A	.030
B	.150	B	.017	B	.014
a	.041	b	.032	c	.016

a − c = .041 − .016 = .025 C.O.D. sample

b − c = .032 − .016 = .016

.016 ÷ 1.6 = .010 C.O.D. standard

$$\frac{.025}{.010} \times 25 = 62.5 \text{ gamma percent}$$

RESULTS AND DISCUSSION

Standard curve

Figure 2 illustrates a representative standard curve obtained with hydrocortisone as reference standard. The results conformed to the Beer-Lambert Law.

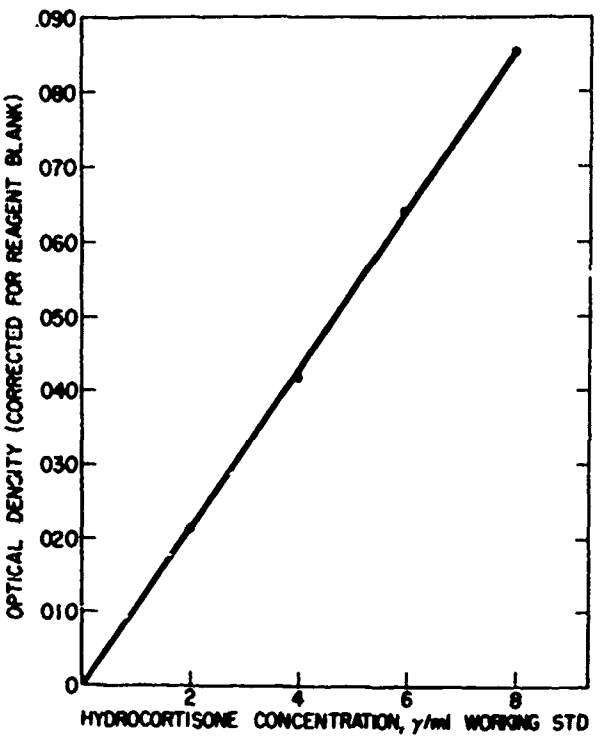

FIGURE 2

A typical standard curve showing the relation between intensity of color and quantity of 17-hydroxycorticoids.

Precision

The precision of the method based on the average difference between 95 sets of duplicates indicated a standard deviation of 5.48 and a coefficient of variation of 10.38 percent.

Recoveries

Recovery studies were carried out by adding known amounts of hydrocortisone in water either to plasma PFF or to untreated plasma samples. Table V shows that, although essentially quantitative recoveries (mean, 98 percent) were obtained by adding standard hydrocortisone at the PFF stage, relatively poor recoveries (mean, 65 percent) resulted from addition of hydrocortisone to plasma.[6]

[5] Since a 4 ml. aliquot is taken in step 2 of the purification, the final standard solutions contain 1.6 gamma and 3.2 gamma of hydrocortisone, respectively.

[6] When known amounts of hydrocortisone dissolved in butanol were added to the protein-free filtrate in step 4 of the extraction procedure, essentially the same recoveries were obtained as found when the water solution of hydrocortisone was added to the PFF.

TABLE V

Recovery of hydrocortisone (compound F) added to plasma or plasma extracts

Sample No.	F present in sample*	F added to plasma	F added to protein-free filtrate	F found*	F recovered (percent)
	(gamma)				
1	2.66	2.0	0	2.85	61
2	2.66	4.0	0	4.70	71
3	2.66	6.0	0	5.10	59
4	2.27	2.0	0	3.04	71
5	2.27	4.0	0	4.32	69
6	2.27	6.0	0	4.82	58
7	3.38	0	2.0	5.46	101
8	3.38	0	4.0	7.28	99
9	3.38	0	6.0	8.36	89
10	2.80	0	2.0	4.80	100
11	2.80	0	4.0	6.65	96
12	2.80	0	6.0	8.70	99

*Each value mean of 2 determinations.

TABLE VI

Plasma levels of total 17-hydroxycorticoids in young adult males sampled at 8 a.m.

Subject No.	17-OHCS (gamma percent)	Subject No.	17-OHCS (gamma percent)	Subject No.	17-OHCS (gamma percent)
1	32.2	19	18.8	37	31.8
2	38.2	20	56.8	38	41.0
3	31.0	21	50.0	39	41.0
4	22.0	22	79.2	40	37.5
5	32.2	23	41.8	41	30.0
6	26.5	24	27.0	42	60.0
7	20.5	25	70.8	43	72.5
8	22.0	26	50.0	44	58.5
9	29.2	27	27.4	45	31.8
10	27.0	28	29.2	46	38.2
11	54.2	29	60.5	47	46.8
12	39.5	30	72.8	48	23.2
13	31.2	31	56.8	49	45.0
14	31.2	32	44.9	50	55.2
15	29.2	33	56.8	51	9.0
16	10.5	34	36.2	52	30.0
17	54.2	35	45.5		
18	20.8	36	63.8		

The Italian workers (6) reported recoveries of 95 to 101 percent when cortisone in butanol was added during either the extraction or the drying phase. Reddy et al. (4) reported recoveries ranging from 94 to 120 percent when aqueous cortisone was added to plasma before preparing the protein-free filtrate. In neither case was it readily apparent at exactly which step in the procedure the standard solution was added.

The reason for the low recoveries on adding hydrocortisone at step 1 of the present study was not clear. The loss of synthetic steroid, evidently into the protein precipitate, is currently under investigation.

Physiologic levels

A series of 52 male subjects, age range 18 to 22 years, was studied at 8 a.m., using the procedure described in this paper. A range of 9 to 82 gamma percent was found (table VI), with a mean of 41.2 gamma percent. Reddy et al. (4) recorded a range of 28 to 78 gamma percent, with a mean of 46 gamma percent, in 6 subjects studied at 8:30 a.m. The values of Brancaccio et al. (6) on 59 subjects varied between 12 and 66 gamma percent, with a mean of 34 gamma percent; the time of blood collection was not recorded.

SUMMARY

A procedure evolved from modifications of existing technics for the estimation of total 17-hydroxycorticoids in blood has been described. Data are also presented on the precision of the method, recoveries of added hydrocortisone, and values obtained in a series of young adult males.

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